AD-A063 898 GEORGIA STATE UNIV ATLANTA DEPT OF BIOLOGY F/G 6/3
BIODEGRADATION OF OIL POLLUTANTS BY YEASTS AND YEAST-LIKE FUNGI--ETC(U)
JAN 78 D G AHEARN, N H BERNER N00014-71-C-045
UNCLASSIFIED GSU-523 NL

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DATE
FILMED
4-79
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Security Classification

DEC 27 1978 bb

DOCUMENT CONTROL DATA - R & D

Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY (Corporate author)

Georgia State University
Atlanta, Georgia 30303

LEVEL II

2a. REPORT SECURITY CLASSIFICATION

Unlimited

2b. GROUP

(2) Sc

(6) REPORT TITLE

BIODEGRADATION OF OIL POLLUTANTS BY YEASTS AND YEAST-LIKE FUNGI

(9) DESCRIPTIVE NOTES (Type of report and inclusive dates)

Final Rept. December 1971 — December 1977

(10) AUTHOR(S) (First name, middle initial, last name)

D. G. Ahearn and N. H. Berner

(11)

6. REPORT DATE

31 Jan 1978

(12) 45 p.

7a. TOTAL NO. OF PAGES

42

7b. NO. OF REFS

29

8a. CONTRACT OR GRANT NO.

(15) N00014-71-C-0145

8b. PROJECT NO.

205-003

8c.

8d.

9a. ORIGINATOR'S REPORT NUMBER(S)

(14) GSU-523

9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)

10. DISTRIBUTION STATEMENT

Unlimited

This document has been approved for public release and sale; its distribution is unlimited.

DDC RECEIVED JAN 30 1979 C

11. SUPPLEMENTARY NOTES

12. SPONSORING MILITARY ACTIVITY

ONR

13. ABSTRACT

In exploring the feasibility of the use of microbial systems for the facilitated biodegradation of waste oils, yeasts and yeast-like fungi from marine, freshwater and terrestrial sources were screened for their ability to utilize hydrocarbons. Mixed cultures of selected fungi, mainly isolates of Candida lipolytica and C. maltosa grew in oil-enriched freshwater or seawater systems fortified with ammonium sulfate or urea. Neither of these yeasts demonstrated capacities for pathogenicity. In estuarine test plots, both species showed affinity for hydrocarbon substrates and did not spread into adjacent pristine habitats. Straight-chained hydrocarbons (C9-C18) were primary substrates, but napthalene and biphenyl content of a synthetic crude decreased concomitantly with utilization of alkanes. Actively growing cells rapidly emulsified light crudes and their refinery fractions, but spent culture broths and dead cells also had emulsifying capacity. Candida lipolytica, but not C. maltosa produced inducible extracellular proteinases. The use of yeasts in mixed culture systems for facilitating the biodegradation of spent oil in confined systems is recommended.

AD A063898

DD FORM 1 NOV 65 1473 (PAGE 1) PLATE NO. 21856

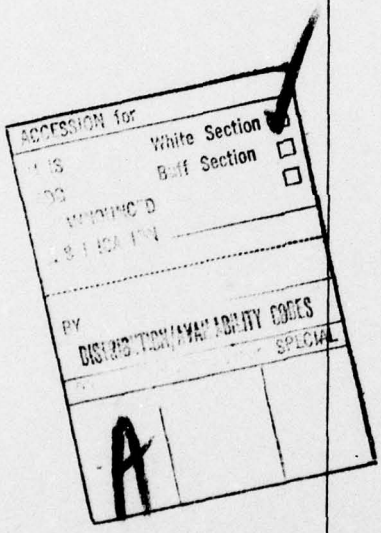
S/N 0102-014-6600

Security Classification

DDC FILE COPY

498 242

14 KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
Candida lipolytica						
Candida maltosa						
Cladosporium resinae						
extracellular proteinases						
oil degradation						
oil emulsification						



OFFICE OF NAVAL RESEARCH

Contract #N00014-77-C-0145

Work Unit No. NR 205-003

FINAL REPORT

BIODEGRADATION OF OIL POLLUTANTS BY YEASTS AND YEAST-LIKE FUNGI

by

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31 January 1978

This research was supported in part by the Office of Naval Research, Microbiology Program, Naval Biology Project, under contract, #N00014-71-C-0145, NR 205-003.

SCOPE OF WORK

Over 1000 fungal representatives from both oil-polluted and pristine terrestrial, freshwater, estuarine and marine sites, as well as from contaminated aircraft fuel systems were isolated during the period 1971-1978. Additional fungi were obtained from the culture collections of the Department of Biology, Georgia State University, and the Department of Food Science, Louisiana State University, Baton Rouge. All of these yeasts and filamentous fungi were tested for their ability to utilize hydrocarbons as sole source of carbon for growth. Selected isolates were examined for their survival and interactions in the environment, pathogenicity for guppies, mice and rabbits, and production of emusifiers and extracellular proteinases. The primary objective was to determine the feasibility of using yeast and yeast-like fungi in facilitating the biodegradation of oil.

METHODS

Hydrocarbon assimilation

Oil assimilation capacity of the more than 1000 fungal isolates was assessed by visual determination of growth in a medium supplemented either with approximately 2% (v/v) Louisiana crude oil or with an equivalent amount (w/v) of the more viscous oils (Tia Juana, Venezuela or Mississippi).

Selected fungi were examined also for utilization of the distillation fractions of crude oil, mixtures of hydrocarbons, a "synthetic crude oil," and various pure hydrocarbons. Cells for inoculum were grown in YNB broth with 0.01% glucose for 24 to 48 hours to accomplish carbohydrate depletion. Of this growth, 0.05 ml was used to inoculate each hydrocarbon utilization

test. Unless otherwise specified, all cultures were incubated at 22 to 26 C on a roller drum set at an angle of 80 and a speed of 40 to 50 rpm. The more viscous hydrocarbons were not sterilized; uninoculated controls were incubated to assess any possible contamination. Visual determination of growth was recorded using a 0-3 scale; zero indicated the absence of growth and three indicated maximal growth as compared to glucose control. The cultures were examined microscopically at various stages during the incubation period.

Oxygen consumption

Oxygen consumption was determined by a modification of the technique of Tool (1967) employing a Hach manometric biochemical oxygen demand (BOD) apparatus, Model 2173. Inocula for the respiration studies contained 4×10^7 to 6×10^7 cells from 24 hour YNB broth cultures with 0.5% glucose. Respiration bottles contained approximately 1.0% hydrocarbon (v/v) and 0.01% yeast extract (Difco) or 0.67% yeast nitrogen base (Difco) in distilled water or seawater. Net oxygen consumption was determined after 72 hours at 6, 20, and 30 C. Although usual BOD procedures require no mixing of bottle contents during the incubation period (American Public Health Association et al. 1971), the procedure used required constant agitation with stirring initiated before the addition of hydrocarbon. Upon completion of the experiment, oily samples from the respiration bottles were streaked on isolation agars to check for culture purity. Control respiration bottles contained either hydrocarbon and yeast extract, inoculum and yeast extract, hydrocarbon only, inoculum only,

or yeast extract only. Net oxygen consumption (mg/l) with 1.0% (v/v) crude oil as substrate was determined by subtracting both endogenous respiration and oxygen consumed in auto-oxidation.

Hydrocarbon Utilization

Selected cultures grown on hydrocarbon substrates were extracted with hexane. The extract was fractionated by column chromatography on an alumina/silica gel column (2/15) (1 cm d x 30 cm). Fractions classed as alkane, aromatic, and asphaltene were eluted with 50 ml portions of hexane, benzene and 1/1 $CHCL_3$-MeOH respectively. All microbial cultures were compared to uninoculated controls incubated and extracted under the same conditions. Gravimetric determinations were made on the residual of both aromatic and asphaltic fractions and the alkane fraction was divided into 2 portions, one analyzed by gas chromatography (GLC), the other by gravimetric methods.

Samples for gas chromatographic analysis were extracted with petroleum ether (2:1). Analysis was done with a flame ionization detector and a Varian Aerograph 2600 with 0.64 cm x 1.8 glass column with 2% SE-30 on Gas/Chrome Q 100/120. The utilization of a synthetic crude oil (Table 1) by selected yeasts was determined by gas chromatographic analyses indicating the percent recovery of individual components as compared to uninoculated controls.

Pathogenicity studies

Pathogenicity studies were conducted with selected strains to assure their safety for man.

Table 1

Crude Oil Composition

Item	Amount	Weight (gm)	Moles	Mole Ratio to Hexadecane
Cyclohexane	1.2 ml	.94	.001	.39
Methylcyclohexane	2.6 ml	2.00	.020	.71
Ethyl benzene	3.9 ml	3.37	.032	1.14
Naphthalene	4.8 gm	4.80	.038	1.36
Biphenyl	4.8 gm	4.80	.031	1.11
Tetradecane	8.5 ml	6.48	.032	1.14
Hexadecane	8.3 ml	6.55	.028	1.00
Eicosane	4.8 gm	4.80	.017	.61

Studies with mice. Cells for inocula were grown in Sabouraud's dextrose broth (Difco) at room temperature for 20 to 24 h with constant agitation. Yeast cells were harvested by centrifuging at approximately 2/3 log phase of growth and washed three times in 0.15 M Sorenson's phosphate buffered saline (PBS). The inocula were adjusted to the desired cell density with a standard curve (595 nm, Bausch & Lomb Spectronic 20).

The LD_{50} for C. albicans for cortisone-treated mice was 5.6×10^4 colony forming units (cfu) and for untreated animals 1.35×10^6 cfu. Inocula of C. albicans of 10^7 and 10^6 cfu were used to challenge the untreated cortisone-treated mice, respectively, to obtain infection of all animals. Inocula of the other yeast species contained 10^7 cfu for all test animals (higher cell concentrations resulted in some deaths within 24 h by occulsion of blood vessels).

Animals. Female Swiss Webster pathogen-free mice (18-20 g, Hilltop Lab Animals) were used in all experiments. Animals were housed in plastic cages and supplied with Purina Lab Chow and water. The 640 mice challenged with yeasts were divided into two test groups: mice in one group received 1.0 mg of cortisone daily (0.1 ml suspension of cortisone acetate, Merck Sharp & Dohme, lot #2568T) intraperitoneally (IP) for 3 days before and 5 days after they were inoculated with yeast; mice in the other group received 0.85% saline IP in the same manner. The mice were inoculated intravenously (IV) in the lateral tail vein with a 0.1 ml suspension of the appropriate inoculum density. A third group of 80 control animals received cortisone IP, 0.1 ml saline IV, and no yeast.

Dead mice and those killed when moribund were necropsied and their kidneys, brains, hearts, livers, and spleens were removed for histopathological examination. At 6 day intervals after inoculation (PI), the most vigorous mice from each group were killed by cervical dislocation and cfu of yeast in the tissue were determined. Organs were removed asceptically and homogenized separately in 3 ml of PBS in 15 ml Ten Broek homogenizers. The tissue suspensions were serially diluted, cultured (pour plates of Mycological agar with chloramphenicol, 250 mg/L), and incubated at 30°C for 2-3 days and then at 22-24°C for 2-3 days before yeasts were counted. The remaining mice were killed 24-30 days PI, and their tissues were collected for yeast quantitation or histopathology. For histopathologic examination, tissues were fixed in 10% phosphate buffered formalin, embedded in paraffin, step-sectioned at 5 μm, and stained with hematoxylin-eosin, Gomori's methenamine silver, and the latter with hematoxylin-eosin counterstain techniques. Tissue sections containing known noncarminophilic fungi were included as positive stain control.

Rabbits. New Zealand white rabbits, 2-4 kg, and pathogen-free rabbits 1-3 kg were used. Rabbits were inoculated IV with 5×10^6 to 1×10^8 cells. Five rabbits inoculated IV with C. maltosa which showed no signs of infection at 14 days were reinoculated at 4-6 day intervals over an additional 28 days. The pathogen-free rabbits included a group treated with cortisone acetate, 5 mg daily for 3 days prior to inoculation with the yeast and for two days following the day of yeast inoculation.

Guppies. Aquaria containing five gallons of freshwater and 1% v/v of two crudes (Louisiana and Mississippi) and with and without 4×10^9 actively growing yeast cells were stocked with five guppies (Lesbistes reticulalus). Survival and feeding of the fish was monitored over a 60-day period.

Proteolysis

Thirty-six cultures of C. lipolytica and two homothallic isolates of its perfect stage Saccharomycopsis lipolytica (Wickerham, Kurtzman et Herman) Yarrow were examined for their ability to produce extra-cellular caseinolysis. The isolates included S. lipolytica 37-1, a yeast used extensively in our hydrocarbon assimilation studies. Other isolates of C. lipolytica, each from different samples, were from liquid soap dispensors (3 cultures), the North Sea (one culture), hydrocarbon storage tanks and aircraft fuel tanks (18 cultures) and rancid yogurt (2 cultures). Caseinolysis of all cultures was determined by clearing of casein agar within 5 days with incubation at 20°C. Preliminary steps in purification and characterization of the proteinases were undertaken (see Publications on Contract for details of procedures).

RESULTS

Hydrocarbon Assimilation

Of a total of 449 yeasts isolated and identified from sites chronically polluted with oil, over 90% of the cultures assimilated Louisiana crude oil as a sole source of carbon. Good growth also was observed on kerosene and light and heavy gas oils. Less than 30% of random isolates from non-oiled regions gave good growth with crude oil as a sole source of carbon. Representative isolates readily assimilated alkanes from C9 to C18 (Table 2), but (except JK29 which showed traces of growth) failed to grow in the presence of C7, C6 or C5 alkanes. The most rapid growth was exhibited by a strain of C. lipolytica 37-1. One strain, C. tropicalis NB2 gave sparse growth on octane, 1-octene and 1 and 2-octene vapors. Only Itersonilia sp. JK29 showed good growth on octane; selected colonies also grew on 1 and 2-octene vapors. The purity of the reagent grade C8 compounds was confirmed by examination of their IR, NMR and GC spectra.

The relative degree of growth of selected strains on various crudes is indicated in Table 3. In general, the less viscous Louisiana oil supported the most vigorous yeast growth and was most readily emulsified. More viscous crudes of higher asphalt content supported fair growth of most strains after 10 days, but the degree of emulsification was relatively poor. There was a general tendency for the yeasts to grow better in sea water than in fresh water media.

Table 2

Growth of Yeasts on Alkanes (2%^{v}/v) in YNB Broth

Species	C7	C8	C9	C10	C12	C14	C16	C18
C. lipolytica 37-1	0[a]	0	3	3	4	4	4	4
C. guilliermondii W30	0	0	1	3	3	3	3	3
C. parapsilosis GM181	0	0	1	3	3	3	4	3
C. tropicalis W12B	0	+[b]	3	3	4	4	4	3
R. toruloides W9	0	0	2	2	2	3	3	3
T. cutaneum GM180	0	0	2	3	4	3	4	4
Itersonilia sp. JK29	+[b]	++[b]	2	2	2	2	2	2
T. capitatum W41	0	+[b]	2	2	3	3	3	3

[a] Relative degree of growth after three days incubation: 0 to 4, negative to maximal (compared with glucose control).

[b] No growth at 2% but growth in the presence of vapors: + slight, ++ good (similar results obtained in 3 repeated tests).

Table 3

Growth of Yeasts on Crude Oils in YNB Broth

Species	Louisiana		Tia Juana		Venezuela		Mississippi	
	FW[a]	SW	FW	SW	FW	SW	FW	SW
C. lipolytica 37-1	4[b]	4	1	1	1	1	0	0
C. tropicalis NB2	3	3	2	1	1	1	1	1
C. tropicalis W12B	3	2	0	2	0	2	1	1
C. parapsilosis GM 131	2	2	0	1	0	1	0	1
T. capitatum SA100	4	3	1	2	0	0	0	1

[a] FW (fresh water), SW (sea water).

[b] Relative degree of growth after three days incubation: 0 to 4, negative to maximal.

Most yeasts isolates grew within three days with Louisiana crude oil as the sole source of carbon. The heaviest growth on Louisiana crude oil after three days incubation was given by strains of C. lipolytica, C. maltosa, C. tropicalis, and Trichosporon fermentans. In contrast to most yeasts tested, both C. maltosa R42 and AJ4476 and C. lipolytica 37-1 produced weak growth on Tia Juana crude oil by ten days. Generally, growth of yeasts with either Tia Juana, Venezuela, or Mississippi crude oil as substrate was minimal and evident only after twenty days incubation. Candida maltosa R42 and C. lipolytica 37-1, in particular, emulsified the crude oils; both the cell-free, spent-culture broths and whole cells showed emulsifying properties.

Strains of C. tropicalis W12B, NB2, Trichosporon cutaneum GM180 and T. capitatum W41 grew well in the presence of vapors of cyclohexane, cyclohexene, thiophene and toluene. When inocula for pour plates were depleted for an additional 12 hours at 5°C in distilled water prior to exposure to the hydrocarbon vapors, growth was negligible. In respiration studies in the presence of thiopene-2-carboxylic acid and glucose, the rate of oxygen consumption was less than that obtained with glucose as the sole substrate.

Filamentous fungi were readily obtained when fuel samples from aircraft and fuel storage tanks were filtered and the filter placed on solid media. Few isolates were obtained by swabbing the fuel directly onto isolation agars. Water agar and Yeast Nitrogen Base (YNB, Difco) agar yielded the greater densities of fungal isolates. Bacteria (Corynebacterium sp., Pseudomonas sp., Bacillus sp.) and sparse yeast growth of Candida guilliermondii, C. parapsilosis, C. tropicalis, Trichosporon cutaneum, Cryptococcus laurentii and

Cryptococcus albidus developed most commonly on Mycological (Difco) agar with chloramphenicol. Broth cultures gave rapid growth of a mixture of bacteria and yeast with apparent suppression of filamentous fungi. Cladosporium resinae, frequently in combination with species of Fusarium, Cephalosporium and Penicillium, as well as Aureobasidium pullulans, was isolated from 18 of 19 jet aircraft fuel systems and 8 of 13 field storage tank bottoms. Multiple culturings and microscopic observations indicated that C. resinae was the major filamentous fungal contaminant. Plating of loose debris from the aircraft fuel tanks consistently yielded C. resinae occasionally in association with the perfect state of a Fusarium (probably Gibberella sp.), Alternaria sp. and Helminthosporium sp.

Representative isolates of C. resinae obtained from contaminated jet fuel used creosote and South Louisiana crude oil as sole sources of carbon for growth. In all cases the growth observed was no more than 50% that of dextrose controls. In commercial aviation jet fuel with a water content ranging from less than 1% to greater than 98%, growth of representative isolates occurred at all concentrations, even in controls to which water had not been experimentally added (i.e., containing dissolved water and water of condensation). In tubes with added water growth was noted within one month, whereas from 12 to 18 months was required for visual detection of C. resinae cultured in jet fuel without direct addition of water. This growth occurred submerged within the fuel, attached tightly to the glass wall or most often to edges of submerged glass chips. The attached fungal growth was hydrophilic and gradually surrounded itself with a water layer. In smooth test tubes incubated with agitation, growth occurred at the water-fuel interphase. In statis cultures C. resinae grew first from pellicle-like growth at the fuel/water

interphase into the fuel phase. In etched test tubes or in tubes with glass chips growth occurred also in the fuel phase firmly attached to the etched ring or to the roughened edges of the chips. Where scratched glass slides were employed, small fungal colonies, starlike in appearance, extended almost to the top of the fuel phase.

In the presence of concentrations of the biocide Biobor JF up to 1:4000 (greater than 10X recommended levels) and with 20% added water, C. resinae did not grow but remained viable for up to six weeks. In the presence of Biobor JF and water contents over 20%, C. resinae produced slow atypical growth with numerous large chlamydospore-like cells. The fungus remained viable for up to 18 months. The anti-icer PFA 55MB at all tested concentrations (0.03 to 0.13%) completely inhibited the growth of C. resinae when the water content was no more than 10% (v/v), and no viable cells were detected after 3 months.

In static cultures, both Fusarium sp. and Penicillium sp. grew profusely in the water phase and only sparsely at the jet fuel/water interphase. Only when attached to scratched glass surfaces was the Fusarium sp. observed in the fuel phase. Inocula of C. resinae, Fusarium sp. and Penicillium sp. placed on glass cover slips under jet fuel showed profuse sporulation within the flattened inoculation drop but only minimal extention into the jet fuel within three months. Short hyphae floating in the jet fuel produced yeast-like buds without typical conidiophore structures; plating these hyphae yielded only C. resinae.

Periodic observations indicated that C. resinae survived for up to three years in sealed jars of jet fuel samples from aircraft wing tanks as well as in samples from storage tanks. After three years, no viable cells could be found.

Oxygen Uptake

Representative yeast isolates from an asphalt refinery, grown on glucose, had similar net rates of oxygen uptake with hexadecane and glucose as substrates (Table 4). Cells of selected strains grown on glucose demonstrated rapid oxygen consumption within 6 hours after being introduced into YNB broth containing Louisiana crude oil. The relative amounts of oxygen consumed after 3 days incubation could be correlated with the visual estimates of growth (Table 5). Microscopic and cultural examinations of the flasks after 3 days demonstrated almost pure cultures of yeasts. When the cultures were permitted to stand over a 10 day period the oil surface films became coated with yeast colonies. In water the oil eventually broke down into distinctly colored (brown and black) globules. With yeast growth the brown film and globules eventually disappeared from the culture broth leaving only black tar-like globules as a residue.

Oxygen consumption by Candida lipolytica 37-1 at 20 C with various crude oils as substrate was compared in both distilled water and filtered seawater (Table 6). Significant oxygen consumption within a 72-hour period was obtained only with Louisiana or Tia Juana crude oil as substrate. No essential difference in results obtained with distilled water or seawater was noted.

Table 4

Oxygen Consumption of Yeasts
from Refinery Site on Hexadecane (10% v/v)

Species	Substrate	
	Glucose	Hexadecane
C. lipolytica A19	46[a]	50
C. tropicalis W25	28	32
C. guilliermondii W30	14.8	13.8
T. capitatum W12B	24.2	28.6

[a]Net QO_2 after 2 hours at 30 C, air as gaseous phase: average of duplicate tests + 3.3. Cells grown for 48 hours on YNB with 0.5% glucose and washed twice in cold phosphate buffer. Flasks contained hexadecane, phosphate buffer pH 5.8 and 1.0 ml suspension of cells.

Table 5

Oxygen Consumption and Growth of
Yeasts on Louisiana Crude Oil

Species	Oxygen Consumed[a]	Growth
Candida lipolytica 37-1	360	4
Candida maltosa R-42	250	3
Candida parapsilosis GM181	60	1
Trichosporon capitatum 1A	32	1

[a]Net oxygen consumed after 3 days at 20 C, calculated as mg/L.

Table 6

Oxygen Consumption by Candida lipolytica 37-1 after 72 Hours at 20 C with Crude Oil as Substrate[a]

Crude Oil	Distilled Water	Filtered Seawater
Louisiana	352[b]	355
Tia Juana	175	215
Venezuela	10	10
Mississippi	10	10

[a] 1.0% crude oil with 0.01% yeast extract added.

[b] Mg oxygen/l, average of 3 repeat tests.

The oxygen consumption at 72 hours with 1.0% Louisiana crude oil as substrate for C. lipolytica 37-1 and C. maltosa R42 was determined at 6, 20, and 30 C (Table 7). Both yeasts had scant oxygen uptake at 6 and highest activity at 20 C. The maximum growth temperature of C. maltosa R42 on Mycological (Difco) agar was about 42 C; this yeast did not grow at 45 C. Candida lipolytica 37-1, unlike most isolates of this species (Lodder 1970) grew rapidly at 30 C and produced only slight growth at 37 C.

Emulsification of Oil

Microorganisms selected from the predominant microflora of the oiled sediments in the estuarine environment were examined for their ability to grow with Louisiana crude oil as a carbon source (Table 8). Of the representative microorganisms, thirteen of the bacteria and three of the yeasts produced some emulsification, but only one bacterium and one yeast gave significant growth with marked emulsification of oil. The yeast Candida sp. MS-309 gave little evidence of utilization by gravimetric analysis. Gas chromatographic analysis however indicated that C11-C13 alkanes accumulated in the culture system.

Two hydrocarbonoclastic yeasts C. lipolytica 37-1 isolated from a frankfurter and C. maltosa R42 obtained from a freshwater holding pond of an asphalt refinery were compared with an isolate of P. ohmeri from the marsh sediments. The culture of Pichia was selected because of its active growth on crude with oil emulsification. The two non-marsh cultures showed greater growth, emulsification, and oxygen uptake on crude oil than the marsh yeast. In cultures with pure hydrocarbons as substrates, C. lipolytica and C. maltosa utilized n-alkanes from C9-C18; C. maltosa also showed some growth on octane. Gas chromatographic analyses demonstrated that both yeasts consumed up to 90% of hexadecane in a culture system within seven days.

Table 7

Oxygen Consumption by Candida lipolytica 37-1 and Candida maltosa R42 after 72 hours with Louisiana Crude Oil as Substrate[a]

Temperature C	37-1		R42		Uninoculated (auto-oxidation)
	Oil	Control[b]	Oil	Control[b]	
6	40[c]	0	25	0	0
20	352	40	313	45	35
30	315	30	270	25	30

[a] 1.0% crude oil with 0.01% yeast extract added, distilled water.

[b] In 0.01% yeast extract, distilled water, no crude oil.

[c] Mg oxygen/l, agerage of 3 repeat tests; ± 25 mg O_2/l at 20 and 30 C, ± 5 at 6 C.

Table 8

Hydrocarbonoclastic Activity of
Representative Estuarine Microorganisms[a]

Total Isolates Examined	Growth on Crude			Emulsification
	Good	Weak	Negligible	
Bacteria 41	1	20	20	1
Yeast 27	3	7	17	1
Fungi 12	0	4	8	0

[a]Seven days growth, shaken culture in basal salts with 2.0% Louisiana crude oil.

Tests were conducted to compare the emulsification of Mississippi and Louisiana crude oils by the yeasts C. maltosa R42 and C. lipolytica 37-1. Live cells, dead cells (autoclaved), and lyophilized cells in concentrations of 1×10^{10}, 1×10^{12}, 1×10^{13}, 1×10^{14}, and 1×10^{15} were added to test tubes (18mm) containing 9.9 ml of sterile tap water and 0.1 ml of crude oil. The various yeast-oil mixtures and control tubes were agitated at 44 rpm on a roller drum at 25 C for one month. Visual observations were made weekly to record the degree of oil emulsification. All tests were run in triplicate. Samples were taken from tubes which displayed the best emulsification of oil after a period of one month. The diameters of 25 oil droplets from each tube were measured microscopically (Table 9).

The mean diameters of oil droplets of both Louisiana and Mississippi crude oils generally decreased with an increased concentration of yeast cells. In general, live yeast cells enhanced the formation of smaller oil droplets than did either dead or lyophilized cells. The adhesion of crude oil to glass test tubes or to wood paddles decreased immediately upon addition of live, dead, or lyophilized yeast cells. This dispersive effect was enhanced by increased concentrations of cells. Emulsification of the crude oil in test tubes inoculated with live yeasts, as determined by weekly visual observations, increased with time and yeast growth over a one month period. This same effect was noted in the aquaria which were enriched with either of the two crude oils and live yeasts. Cells of C. lipolytica 37-1 were more effective dispersants of Louisiana crude oil, whereas cells of C. maltosa R42 gave visual evidence of greater emulsification of the more

Table 9

Average diameter of crude oil droplets emulsified by yeasts[a]

	Louisiana Crude				Mississippi Curde			
	C. maltosa		C. lipolytica		C. maltosa		C. lipolytica	
Cell Type	Diam[b]	Range[c]	Diam	Range	Diam	Range	Diam	Range
Autoclaved	101	20-580	82	20-385	63	10-305	55	15-230
Lyophilized	80	15-405	58	10-250	44	10-200	51	10-530
Live	83	15-450	51	10-180	54	10-515	94	10-335
Control (no yeast)	165	50-925			574	20-2870		

[a]Measurements in micrometers by ocular micrometer; sample from suspended material after agitation for 25 days.

viscous Mississippi crude oil. In general, Mississippi crude was not as dispersed as the Louisiana crude, although the average size of measurable oil droplets of Mississippi crude oil was smaller than those of Louisiana crude. Many more large, hardened globules above 1 mm in diameter were present in the tests involving Mississippi crude than in those with Louisiana crude.

The mixture of either crude oil with either yeast was more effective in reducing the adhesiveness of the oil to glass than was the mixture of Polycomplex A-11 (Guardian Chemical Corp., Happange, N. Y.) dispersant with either oil. This effect was noted in both test tubes and aquaria. Microscopic examination of agitated cultures showed that these yeasts coated the sub-surface oil globules with budding cells, whereas in surface slicks in non-agitated systems, mats of hyphae were formed within and upon the oil layers. Similar growth results were obtained from media prepared with either freshwater of seawater. Rhodosporidium toruloides CM183 and numerous other red yeasts (species of Rhodosporidium and Rhodotorula) isolated from oil-polluted areas utilized components of crude oil for growth and energy, but never with amounts of growth or emulsification comparable to those of the species of Candida.

Freshwater Field Studies

Selected fungi from the initial screening for hydrocarbonoclastic abilities served as inocula for freshwater tests which were designed to simulate oil spills of lakes or ponds. These tests allowed weathering of the oils with natural temperature fluctuations. Survival of the seed cultures was

monitored. Early environmental studies involved mixed inocula of the hydrocarbon assimilating fungi.

Test series 1. A mixture of eight fungi (Candida tropicalis NB2 and W12B, C. lipolytica 37-1, C. parapsilosis GM181, Trichosporon fermentans SA100, Cladosporium resinae SA300, Trichosporon cutaneum GM180, Rhodosporidium toruloides GM183, and Itersonilia sp. JK29) was introduced into two separate test systems in freshwater obtained from an asphalt refinery holding pond (heavily oil polluted) and from the refinery's drainage system. The water was enriched with Louisiana crude or bunker C oil. The most noticeable effects of the fungi on the oiled water were partial disruption of the surface slick, development of a more evident oil-in-water emulsion, and the formation of matted, irregular hyphal layers at the oil-water interphase. Such phenomena were much less evident in the control containers. Periodic samplings of one test system followed by isolation and identification of yeasts over a five month period demonstrated a gradual decrease in certain populations, the disappearance of a few species, and the establishment of wild fungi in both test and control vats (Table 10). Approximately the same number of fungi were obtained from test buckets with and without enrichment with 25g of $(NH_4)_2SO_4$. Less microbial activity was evident in the bunker C fuel oil systems than in the Louisiana crude. Species of Cladosporium which gave negligible evidence of growth on crude oil in pure culture systems were the predominent isolates from the bunker C field tests (Table 10). Bacteria were present in all test systems, particularly in those enriched with bunker C fuel oil. Neither oil was completely degraded during the five-month test period.

Table 10

Predominant Fungi Recovered from Asphalt Refinery Lake Waters
Enriched with 4% (v/v) Hydrocarbons

	Louisiana Crude Oil						Bunker C Fuel Oil[a]			
	Inoculated			Uninoculated			Inoculated		Uninoculated	
	1 mo.	3 mo.	5 mo.	1 mo.	3 mo.	5 mo.	1 mo.	3 mo.	1 mo.	3 mo.
Yeast										
Candida tropicalis[b]	25[c]	30	40	/[d]	/	5	10	5	/	/
Candida lipolytica[b]	25	25	20	/	/	/	10	5	/	/
Candida parapsilosis[b]	/	/	10	/	/	/	/	/	/	/
Trichosporon fermentans[b]	15	20	10	/	/	/	75	15	/	/
Red Yeast	25	20	13	50	25	25	5	5	50	50
Filamentous fungi										
Cladosporium sp.[b]	5	/	7	/	/	/	/	50	/	/
Cephalosporium sp.	/	5	5	5	25	25	/	5	5	5
Fusarium sp.	/	/	/	5	25	25	/	5	5	5
Penicillium sp.	/	/	/	5	5	10	/	5	5	5
Alternaria sp.	/	/	/	5	15	10	/	/	/	5
Aspergillus sp.	/	/	/	5	5	/	/	/	/	5
Trichoderma viride	/	/	/	/	/	/	/	5	/	5

[a]Bacterial growth predominant in all bunker C buckets.

[b]Organisms seeded into inoculated buckets.

[c]Percent of total fungal isolates.

[d]/ indicates not isolated.

Test series 2. A second series of seed-culture tests involved freshwater from a non-oil-polluted lake; the water was enriched with 4% (v/v) Louisiana crude oil. Containers were placed outdoors and inoculated with a mixture of seven fungi (Cladosporium resinae SA300, Candida parapsilosis GM181, Pichia ohmeri LSU216, C. tropicalis NB2, C. maltosa R42, Trichosporon fermentans SA100, and C. lipolytica 37-1). Samples from test and control systems were taken periodically for twelve months. Within three months, the integrity of the initial oil layer (approximately one centimeter) in each inoculated bucket was disrupted, and there was noticeable evaporation of water. At one year, a film of crude oil was no longer present in the inoculated buckets; instead a thick, spongy pellicle covered the water surface; the pellicle had a black, hardened, asphalt-like upper surface and a brown, spongy underside with much stringy mycelial growth. Thick hyphal strands extended down into the turbid water; many strands sank to the bottom of the containers. Only about 500 ml of water remained. The oil film in the control container was reasonably unaltered, and the water volume remaining was approximately four liters at the end of one year. The uninoculated control (water from the non-oil-polluted lake) showed little evidence of microbial activity. Of the seven species of fungi introduced into the test systems, four species (Trichosporon fermentans, Cladosporium resinae, Candida parapsilosis, and Pichia ohmeri) were never recovered. Candida lipolytica was recovered at the end of the first three weeks but not thereafter. Candida maltosa was obtained on each sampling throughout the year. During this time both control and inoculated buckets were populated with numerous fungi, mainly species of Alternaria, Cephalosporium, Fusarium, and Penicillium.

Estuarine Field Studies

Water, sediment, and vegetation (Spartina alterniflora) samples were taken from the northwestern region of Barataria Bay, Louisiana, at intervals during a two year period and examined for their fungal flora. Densities of proteolytic, cellulolytic, hydrocarbonoclastic, and total heterotrophic bacteria in oiled and non-oiled marsh sites are presented in Table 11. With the addition of crude oil, cellulolytic bacteria appeared to decrease in number, whereas densities of hydrocarbonoclasts increased. A similar trend was noted for bacterial populations at the site of the Martigan Point oil spill. Prior to the introduction of the seed cultures into estuarine waters, species of Pichia, Kluyveromyces, Rhodotorula, and Cryptococcus were found to predominate at the test site. No isolates of these species produced strong growth on hydrocarbons before exposure of the marsh site to oil. In response to oil, total cell populations doubled, and there was a shift in the microflora from species of Pichia and Kluyveromyces to species of Rhodotorula and Trichosporon (Table 12).

A mixture of six hydrocarbonoclastic fungi (Trichosporon fermentans SA100, Cladosporium resinae SA300, Candida tropicalis NB2, C. maltosa R42, C. lipolytica 37-1, and C. parapsilosis GM181) was introduced into estuarian waters in Barataria Bay, Louisiana. Maintenance of the seed plots in the estuary was facilitated by the use of fifty gallon drums with both ends removed and with lateral holes below the water line. These barrels were positioned in the estuarine waters and inoculated with a mixture of either growing cells or lyophilized cells of six fungi, including five yeast and one filamentous isolate. Louisiana crude oil was added to all barrels at

Table 11

Populations of heterotrophic bacteria in oiled and non-oiled marsh sediments

	Concentration of bacteria (log 10/g wet sediment)					
	Jan	Feb	Mar	May	June	July
Airplane Lake Site						
Control						
total	7.72	7.93	8.36	7.99	8.60	7.60
cellulolytic	4.97	6.34	6.15	5.85	4.36	6.20
proteolytic	5.78	6.57	6.45	6.59	6.30	6.45
hydrocarbonoclastic	5.52	4.78	5.36	4.89	3.90	4.23
Oil Plot						
total	7.84	8.11	8.32	7.96	8.18	7.43
cellulolytic	4.22	5.63	5.49	5.04	2.85	5.20
proteolytic	6.18	6.69	6.48	6.39	6.81	6.08
hydrocarbonoclastic	6.52	7.20	6.66	6.50	7.36	5.60
Martigan Point, Oil Spill Site						
Control						
total	--	--	--	7.81	8.70	7.95
cellulolytic	--	--	--	5.45	5.38	4.70
proteolytic	--	--	--	5.60	5.49	5.40
hydrocarbonoclastic	--	--	--	4.84	4.95	4.70
Station No. 1						
total	8.15	8.87	--	9.65	8.30	7.68
cellulolytic	4.98	7.34	--	5.98	4.41	3.85
proteolytic	6.25	7.00	--	6.58	5.28	6.15
hydrocarbonoclastic	6.60	7.73	--	7.04	5.53	6.96

Table 12

Effect of Louisiana crude oil on yeast populations in coastal marsh sediments.

Genera	Per cent of total population Before	After[a]
Pichia/Kluyveromyces	85-100	10-83
Rhodotorula/Rhodosporidium	<10	5-50
Cryptococcus	<15	< 10
Trichosporon/P. ohmeri	<10	12-35
mean population[b]	7150	14772

[a]determined two months after oil addition, analysis of 33 samples in triplicate.
[b]colony forming units per mg wet sediment.

3 to 5 week intervals over an eight month test period. Colony forming units (cfu) of C. maltosa and C. lipolytica constituted approximately 95% of all microorganisms recovered from water samples taken 24 hours after inoculation. A few red yeasts were also evident. No Trichosporon or Cladosporium species were recovered.

After the first sampling, high tides in the fall of the year completely inundated the test barrels; both the seeded crude oil and the top stratum of water were washed away. Samples for fungal identification were taken and Louisiana crude oil again was applied to the plots, but the plots were not reinoculated with yeasts. Analysis of the samples indicated that the indigenous fungi had returned and the seeded Candida species were lost. Of the fungi isolated, approximately 40% of the cfu were species of Pichia, 20% Kluyveromyces, 20% Cryptococcus, 10% red yeasts, and 10% filamentous fungi. Cladosporium species were rarely isolated; those isolates examined were morphologically distinct from the "kerosene fungus" Cladosporium resinae. Almost eight months after the original inoculation, high spring tides washed some of the barrels out to sea. Essentially the same isolations of fungi as those reported earlier were made. Additions of oil during the interim period had not encouraged evident growth of organisms other than the indigenous fungi.

In subsequent seed culture tests in the marsh, only Candida lipolytica 37-1 and C. maltosa R42 were used for inoculation of sediment plots. One hundred ml of Louisiana crude oil was applied to the plots prior to inoculation and then periodically over an eleven month test period. Duplicate samples from the estuarine barrels were filtered through cellulose-ester

membranes (Millipore Filter Corp.) of 0.45 μm porosity, and the membranes were implanted aseptically onto distilled and seawater isolation media. Sediment and vegetation samples from the oiled plots were taken before inoculation and after two months, three months, seven months, and eleven months, with sterile utensils and plated directly onto isolation media. Occasional samples were kept on ice for 5 to 10 hours prior to culturing. Part of each sediment sample was diluted with sterile seawater, vigorously agitated, and the supernatant was filtered through cellulose-ester membranes; the membranes were then aseptically implanted onto isolation media. Control samples were taken from the natural bay waters, sediments, flora, and from oil-seeded, uninoculated barrels and marshland plots. Representative yeasts and filamentous fungi were selected from the isolation plates on the basis of colony morphology and were identified (Table 13). Although the yeasts C. maltosa R42 and C. lipolytica 37-1 were not found in Barataria Bay prior to their inoculation as seed cultures, they persisted for over seven months in oiled plots in the marsh. Candida maltosa was recovered in greater densities from all samples of the oil-soaked plots. Neither yeast was isolated from adjacent non-oiled sites throughout the entire study. The seed yeasts survived the seasons from late fall to late spring or early summer. Representatives of Pichia spartinae with the ability to utilize and emulsify crude oil were isolated after the plots were subjected to oil for at least several months. Microscopic examination of the growth on oil revealed that the yeast produced short chains of aberrant, pseudo-hyphal cells interspersed with numerous chlamydospores. Of the major species

Table 13

Fungi Isolated from Oiled Sediments
Barataria Bay, Louisiana

	Prior to Inoculation	Time After Inoculation of Seed Cultures							
		2 months		3 months		7 months		11 months	
		oiled	control[b]	oiled	control	oiled	control	oiled	control
Seed cultures:									
Candida maltosa R42	0[a]	+	0[c]	+	0	+	0	0	0
C. lipolytica 37-1	0	+	0	+	0	+	0	0	0
Indigenous Fungi:									
Yeast-like									
Rhodotorula-Rhodosporidium sp.	+	+	+	+	+	+	+	+	+
Cryptococcus sp.	+	+	+	+	+	+	+	+	+
Kluyveromyces sp.	+	+	+	+	+	+	+	+	+
Pichia sp.	+	+	+	+	+	+	+	+	+
Trichosporon sp.	+	+	+	+	+	+	+	+	+
Aureobasidium sp.	+	0	+	0	+	0	+	0	+
Filamentous									
Cladosporium sp.	+	0	+	+	+	0	+	+	+
Penicillium sp.	+	0	+	+	+	0	+	0	+
Cephalosporium sp.	+	+	+	+	+	+	+	+	+
Fusarium sp.	+	+	+	+	+	+	+	+	+
Alternaria sp.	+	+	0	0	+	0	+	0	0
Trichoderma viride	0	0	+	0	0	0	+	0	+
Aspergillus sp.	0	0	0	0	0	0	0	+	0

[a] Indicates not isolated, + present.

[b] Control plots, periodically oiled, no inoculum.

[c] Seed cultures introduced in non-oiled sediments were not recovered after 72 hours.

[d] Major species; *C. albidus*, *C. laurentii*, *K. drosophilarum*, *P. spartinae* and *T. cutaneum*.

Guppies survived an average of 11 days in aquaria with Louisiana crude. With the addition of yeasts, the fish survived an average of 12 days. In Mississippi crude, the fish survived an average of 25 days, whereas, with yeasts added, survival was reduced to an average of 13 days. In tanks without oil or yeasts the guppies survived an average of 25 days, whereas with the addition of yeasts the fish survived an average of 28 days.

Extracellular proteinases

Thirty-three isolates of C. lipolytica produced zones of clearing in casein agar within 3 days at 20°C. Culture 37-1 gave the most rapid clearing. Filtrates of broth cultures were proteolytic and showed activity at pH 4.2-4.8 and pH 6.5-7.8. Secretion of the neutral protease was repressed in the presence of glycerol or glucose, both of which supported rapid growth of the organism. Ammonium ions also repressed the secretion of the enzyme. The neutral protease activity (casein as the substrate) was co-purified with esterase activity (benzyloxycarbonyl tyrosine p-nitrophenyl ester) during ammonium sulfate fractionation, chromatography on DEAE-cellulose, and gel filtration on Sephadex G-150. The final enzyme preparation was homogenous as judged by polyacrylamide gel electrophoresis. The molecular weight of the enzyme was estimated to be 38,000 by gel filtration and 38,500 by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The purified enzyme had a pH optimum of 6.8 and was sensitive to phenylethylsulfonylfluoride and ethylenediamine tetraacetate. This dual sensitivity and the pH optimum classified the enzyme as both a neutral metaloprotease and a serine protease. Divalent ions significantly

indigenous to the area, no other hydrocarbonoclastic isolates were found. Filamentous fungi with weak hydrocarbonoclastic properties, including species of Trichosporon (mainly T. cutaneum), Aureobasidium, Penicillium, and Cephalosporium were obtained throughout the study.

Pathogenicity Studies

Candida albicans and Candida tropicalis were used as positive pathogenic controls. All mice that received C. albicans and 18 of the 20 cortisone-treated mice and 10 of the 20 untreated mice challenged with C. tropicalis died between 4 and 20 days after they were inoculated. These yeasts were found in nearly equivalent densities in the kidneys and brains of sacrificed untreated and cortisone-treated mice. Between 6 and 12 days PI brain populations declined, and kidney populations remained approximately constant. Yeasts in densities of <10 to approximately 10^6 cfu were recovered sporadically from the heart, liver, and spleen. Of the mice which survived the test period, all of those treated with cortisone and 9 of 10 of those injected with C. tropicalis yielded yeast cells, mainly from kidney tissues, 30 days PI.

All rabbits inoculated with C. albicans developed symptoms of candidiasis, whereas of those receiving C. tropicalis only cortisone-treated rabbits showed signs of disease.

Candida lipolytica was never recovered from test animals. Candida maltosa (and non-pathogenic controls, C. utilis and Saccharomyces cerevisiae) persisted in the tissues of mice and rabbits for varied periods, but they were non-lethal and no evidence of tissue invasion was detected.

activated protease activity. Dithiothreitol inhibited both protease and esterase activities indicating the presence of a critical disulfide bridge. The electrophoretic nature of the enzyme coupled with the nature of its retention on gels suggested it contained a lipid moiety. This moiety may aid in the emulsification of hydrocarbons by C. lipolytica.

Utilization of Synthetic Crude

Culture C. lipolytica 37-1 and C. maltosa R42 utilized 26.2 and 35.7% of the synthetic crude oil provided as a sole source of energy (1.0% v/v in distilled water). The addition of glycerol or glucose reduced utilization by C. maltosa, but not by C. lipolytica. The utilization of the individual components of the crude is indicated in Table 14. Although the aromatic fractions are reduced concomittantly with alkane utilization, preliminary radiolabel studies have not demonstrated aromatic degradation.

Table 14

Utilization of Synthetic Crude Components
by C. lipolytica and C. maltosa

	Ethyl-benzene	Naphtha-lene	Biphenyl	Tetra-decane	Hexa-decane	Eico-sane
C. maltosa						
Synthetic Crude	29.3*	23.5	17.3	43.1	44.1	43.8
+ Glycerol	17.5	17.6	21.8	31.3	30.7	30.2
+ Glucose	26.7	20.3	23.1	26.2	28.2	27.6
C. lipolytica						
Synthetic Crude	29.4	14.6	34.6	32.9	32.7	35.3
+ Glycerol	27.8	20.0	19.6	36.8	36.1	29.8
+ Glucose	46.7	22.0	21.8	23.6	22.7	23.8

*Per cent utilization compared to an uninoculated control

SUMMARY AND DISCUSSION

The indigenous yeast flora of pristine freshwater and estuarian environments demonstrated limited capacities to utilize crude oils as contrasted with yeasts from sites enriched with petroleum. Only after about a year of exposure to oil did a few representatives of the indigenous flora show hydrocarbonoclastic activity. Hydrocarbonoclastic fungi introduced into oil-soaked habitats did not all survive. Of the fungi used, isolates of C. maltosa and C. lipolytica persisted in both freshwater and estuarine environments enriched with oil for varying periods of time without apparent adverse effects on the ecology of the study sites. Moreover, these yeasts seemed to be localized at the oiled plots. Candida maltosa persisted the longest at both freshwater and estuarine sites. No adverse ecological conditions were observed which could be attributed to addition of yeasts to water containers or sediment plots.

Candida maltosa R42 and C. lipolytica were able to degrade a broad range of alkanes and alkenes (and possibly aromatics) grew and multiplied in both freshwater and seawater and emulsified oils. Neither yeast was lethal nor invasive for mice or rabbits nor produced adverse signs in guppies. Emulsifying properties of the strains appeared to be related to cellular coatings and possibly associated with extracellular secretions.

The data suggests that these yeasts may be used as seed cultures for refinery waste treatment systems or in facilitating the removal of sludge oils from shipboard installations. In such systems, growth conditions could

be partially controlled, and if necessary, nutrient enrichment or surfactants could be used. Although complete utilization of crude oil has not been achieved by any culture system a microbial seed system which emulsified as well as utilized part of the crude oil would still be of practical value in removing oils from contained situations.

LIST OF MAJOR ACCOMPLISHMENTS

Isolation and identification of hydrocarbonoclastic yeasts of potential use in the facilitated biodegradation of oil.

Establishment of target-orientation of selected strains and lack of obvious adverse environmental effects.

Establishment of non-pathogenicity of selected strains and apparent safety for exposure to humans.

Partial characterization of extracellular proteinases of C. lipolytica.

Publication of 29 scientific reports on contract (see attached list).

REPORTS AND PUBLICATIONS ON CONTRACT

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